Physiological factors determining vesicular–arbuscular mycorrhizal formation in host and nonhost Ri T-DNA transformed roots

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Transformed roots of carrot (Umbelliferae) and sugar beet (Chenopodiaceae) were used as model host and nonhost plant, respectively, for the vesicular–arbuscular mycorrhizal fungus *Gigaspora margarita* (Becker & Hall). Rapid growth of hyphae from germinating spores of *G. margarita* and formation of infection units were obtained only in the presence of carrot roots. Root volatiles from both plant species have similar stimulative effects on hyphal growth. However, hyphal growth was stimulated by root exudates of carrot and not by those of sugar beet. These and other results suggest that the nonmycorrhizal roots of sugar beet lack factors that promote mycorrhizal infection rather than producing inhibitory factors. The model used in this study is especially appropriate for further investigations on the recognition mechanisms involved in vesicular–arbuscular mycorrhizal associations.

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Des racines transformées de carotte (Umbelliferae) et de betterave (Chenopodiaceae) ont été utilisées, respectivement, comme modèles de plante-hôte et non-hôte pour le champignon mycorhizien à vésicules et arbuscules *Gigaspora margarita* (Becker & Hall). C'est seulement en présence de racines de carotte qu'ont été obtenues une croissance rapide des hyphes ainsi que la formation d'unités d'infection. Les composés volatiles racinaires des deux espèces végétales ont eu un effet stimulateur similaire sur la croissance des hyphes. Par contre, cette croissance a été stimulée par les exsudats racinaires de la carotte mais pas par ceux de la betterave. Ces résultats et d'autres suggèrent que les racines non-mycorhiziennes de betterave, plutôt qu'elles ne produisent des facteurs qui inhiberaient le champignon, n'en produisent pas qui favorisent l'infection mycorhizienne. Le modèle utilisé dans cette étude est particulièrement approprié pour poursuivre des recherches sur les mécanismes de reconnaissance dans les associations mycorhiziennes à vésicules et arbuscules.

Introduction

Certain vascular plants occurring within the Cruciferae and Chenopodiaceae have often been reported as being nonhosts to vesicular–arbuscular (VA) mycorrhizal fungi. Recently, a review by Tester *et al.* (1987) examined current hypotheses concerning the lack of infection in such nonhosts, including proposed differences in the nature of soluble or volatile factors produced by nonmycorrhizal roots. These root factors could be fungitoxic (Robinson 1972; Hayman *et al.* 1975; Bevege and Bowen 1975; Morley and Mosse 1976; Tommerup 1984; and El-Atrach *et al.* 1989), produced in quantities insufficient for fungal nutrition (Schwab *et al.* 1982, 1984), or else lacking critical factor(s) that control mycorrhizal infection (Glenn *et al.* 1988). An alternative hypothesis was proposed by Tester *et al.* (1987), who suggested that the control of mycorrhizal fungal penetration is exerted during later interactions between the organisms, at the level of the cell wall and (or) middle lamella. An example of such later control of the VA mycorrhizal fungus by a nonmycotrophic plant was recently reported by Allen *et al.* 1989. After being invaded and leading up to arbuscule formation, roots of *Salsola kali* L. (Chenopodiaceae) were observed to develop progressive, incompatible reactions with different VA mycorrhizal fungi.

In the above-mentioned studies, different degrees of mycorrhizal infection of normally nonsusceptible plants were reported, depending on the plant species and environmental conditions. Tester *et al.* (1987) stressed the importance of determining whether such infections are due to nonspecific colonization of old roots or not. Furthermore, they expressed the need to determine if noninfected plants are solely the results of environmental effects.

Investigations on the barriers to infection of nonmycorrhizal plants are important for understanding vesicular–arbuscular mycorrhizal infection processes in general. Such investigations need to discriminate between environmental and intrinsic factors involved in the formation of VA mycorrhizae, using a simple axenic experimental set-up and design. Bécard and Piché (1989*b*) showed *in vitro* that growth of hyphae from germinating spores of *Gigaspora margarita* was considerably stimulated by the synergistic action of root volatiles and exudates using a system based on Ri T-DNA transformed roots. They demonstrated that root CO_2 plays a critical role and hypothesized that a more specific factor(s) in the exudates serves as a signal(s) to promote hyphal growth. We report here the use of Ri T-DNA transformed roots of *Daucus carota* L. (Umbelliferae) and *Beta vulgaris* L. (Chenopodiaceae) as an axenic model system for the study of mycorrhizal and nonmycorrhizal plants, respectively. Using this reproducible sys-

tem, the objective of this paper was to examine further the possibility that these interactions between the two symbionts participate in early recognition processes.

Materials and methods

Root organ culture

A clone of Ri T-DNA transformed root of carrot (*D. carota* L.) (Bécard and Fortin 1988) and one of sugar beet (*B. vulgaris* L.) initiated by Dr. D. A. Tepfer (Institut national de la recherche agronomique, Versailles, France; provided by G. Zahka, University of Copenhagen) were routinely propagated on a minimal medium (Bécard and Fortin 1988) in Petri dishes. A richer modified White's (MW) medium was occasionally used to obtain more prolific root growth (Bécard and Fortin 1988). Root explants from routine culture were prepared as previously described (Bécard and Piché 1989*a*).

Fungal inoculum

Spores of *G. margarita* Becker & Hall (DAOM 194757, Biosystematic Research Centre, Ottawa, Ont.) were recovered from leek pot cultures, purified, surface sterilized, and stored according to the procedure of Bécard and Fortin (1988). A single spore, placed in the middle of a square (9 × 9 cm) Petri dish containing minimal medium, was used as an experimental unit for all experiments. Dishes were sealed with Parafilm, placed vertically, since the germ tube elongates upward as a result of its negatively geotropic mode of growth (Watrud *et al.* 1978), and incubated in the dark at 26°C. Two autoclaved cotton rolls (dental rolls, Healthco DDL, Montreal, Que.) were placed in each Petri dish to absorb excess water.

Fungal growth in presence of roots

Two days after spore germination, roots were placed both perpendicular to and in front of the growing germ tube to initiate a dual culture (Bécard and Piché 1989*a*). Root explants were grown on minimal medium for 3 weeks prior to being used (Fig. 1A).

Fungal growth with different combinations of root exudates and volatiles

Root exudates were added to a Petri dish containing minimal medium by using it to cultivate two root explants for 2 weeks. The roots were placed on a polycarbonate membrane (Nuclepore Corporation, Pleasanton, CA) that was laid on the surface of the agar medium then overlayed with a thin layer of additional agar medium. The roots were removed with the membrane before the spore was introduced.

Root volatiles were provided to the experimental unit by replacing the cover of the Petri dish with the bottom of another containing agar that was used for growing one root. The pair of Petri dishes were held together using Parafilm.

Assessment of fungal growth

The linear growth of hyphae emerging from germinating spores was measured in millimetres using a gridline intersect method described by Bécard and Piché (1989*a*). In the presence of roots, fungal growth was also assessed by counting the total number of infection units that had completed arbuscule formation in the root system of each individual Petri dish. For this purpose, roots were cleared in 10% (w/v) KOH for 10 min, rinsed in water, and stained in 0.15% (w/v) chlorazol black E for 1 h (Brundrett *et al.* 1984). Sugar beet and carrot roots, which had been grown together, were carefully separated under a stereoscopic microscope. Six to 17 replicates were performed for each experimental treatment. The various effects of the treatments on fungal growth were interpreted in terms of extraradical growth curve patterns. However, statistical analyses were performed on the means corresponding to the last data points on the curves to verify (after 2 or 3 weeks depending on the experiments) the significance of the treatments. The Student's *t*-test was used for comparisons in groups of two means, and the multiple comparison Waller–Duncan's test in groups of three means.

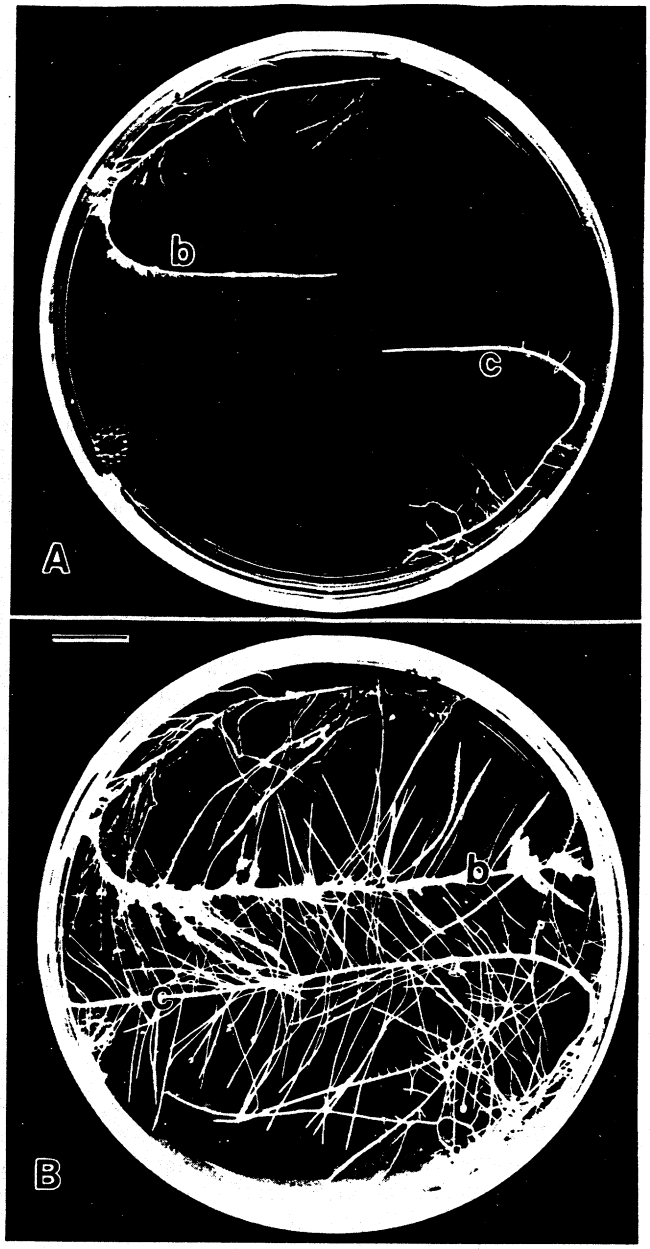

FIG. 1. Root organ culture of Ri T-DNA transformed roots of carrot and sugar beet on a common medium. (A) Initial root explants of carrot (*c*) and beet (*b*). (B) The same two root explants after 12 days of growth in the dark at 26°C. Bar = 2 cm.

Results

Root growth

The transformed roots of carrot and sugar beet had obvious morphological differences (Fig. 1), with the latter being thinner and producing longer and more numerous root hairs. However, root apices of both species elongated at very similar and constant rates, having a mean value of 14 and 15 mm/day, respectively, with a coefficient of variation of 8 and 23%, respectively (n = 10). Both root systems branched profusely (Fig. 1B).

Test for mycorrhizal formation

After 3 weeks of growth, hyphal elongation in the presence of carrot roots was greater (400%) than that in the presence of

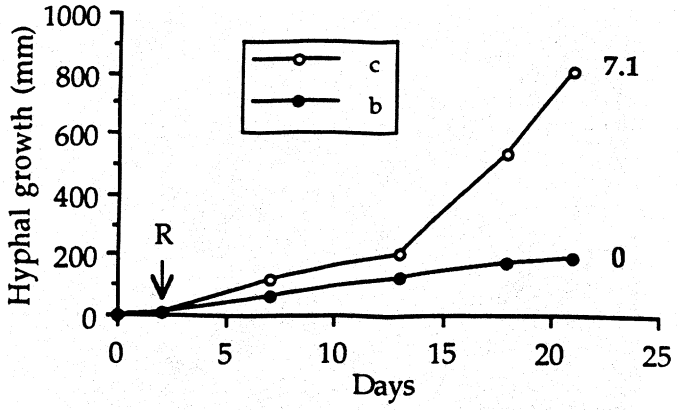

FIG. 2. Average hyphal growth from germinating spores of *G. margarita* in dual culture with a carrot root (*c*) or with a sugar beet root (*b*). The roots (R) were introduced 2 days after spore germination. The average number of infection units on day 21 is indicated beside the respective data points.

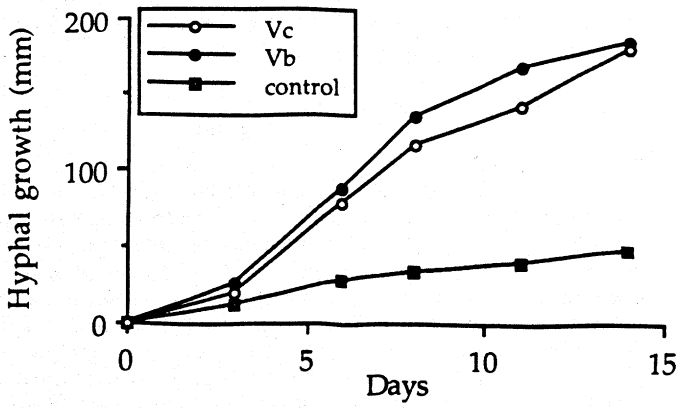

FIG. 3. Average hyphal growth from germinating spores of *G. margarita* in presence of carrot root exudates without root volatiles (control) or with volatiles produced by growing roots of carrot (Vc) and sugar beet (Vb).

sugar beet roots (Fig. 2). An average of 7.1 infection units per Petri dish were formed for carrot roots, but neither an infection unit nor an appressorium was ever found with roots of sugar beet. The difference in final fungal growth was statistically significant (Student's *t*-test, $p < 0.0001$), and the divergence in growth response could rapidly be seen after root introduction.

Effect of root volatiles

Both roots of carrot and those of sugar beet produced volatiles that have a similar and significant (Waller–Duncan test, $p < 0.01$) stimulative effect on hyphal growth (Fig. 3). After 2 weeks of culture in the presence of carrot root exudates, hyphal growth in the presence of these volatiles was much higher (380%) than that in their absence (control).

Effect of root exudates

In the presence of root volatiles of carrot, hyphal growth was significantly greater (270%) in the presence of root exudates from carrot than in the presence of exudates from sugar beet or in the complete absence of any root exudates (Waller–Duncan test, $p < 0.01$)(Fig. 4). Root exudates from sugar beet had neither a stimulative nor a detrimental effect on hyphal growth.

Fungal culture with both types of roots on a common medium

Infection units were found in carrot roots but not in beet roots. Even hyphal attachments on the root surface were not found with the latter. After 3 weeks of culture, hyphal elongation in the presence of both a sugar beet root and a carrot root was significantly (Student's *t*-test, $p = 0.0018$) less (540 mm) than when compared with a Petri dish containing a second carrot root in place of the sugar beet root (960 mm) (Fig. 5). However, covariance analysis showed that hyphal elongation in these two cases was not significantly different ($p = 0.18$) when the number of infection units was used as a covariable. These results indicate that the difference in hyphal elongation can be explained by the difference in the number of infection units obtained in the two treatments rather than by a specific inhibitory effect of beet roots on fungal growth. This interpretation is more logical than the reverse, i.e., that the difference in number of infection units is explained by the difference in hyphal growth, because sugar beet roots competed for the same nutrients as the carrot roots, forcing the latter to spread in the plates at half their potential. It was then expected that half of the number of infection units (4.2) would be found compared with that of the two carrot root systems (8.1) and consequently proportionally reduced extraradical growth, since there is a positive correlation between the two variables.

Discussion

The mycorrhizal status of transformed roots of carrot involves their ability to produce factors that stimulate growth of *G. margarita* during the preinfection stage (Bécard and Piché 1989*b*), to sustain the biotrophic growth of this fungus (Bécard and Piché 1989*a*), and to allow the completion of the fungal life cycle up to sporulation (Bécard and Fortin 1988). The rapid colonization by a single germinating spore led to the formation of an average of 7.1 infection units per carrot root explant in 3 weeks. Using the same protocol, the transformed roots of sugar beet remained uninfected.

No difference was found between the root volatiles produced by these species in their ability to stimulate hyphal growth. This result is expected, since Bécard and Piché (1989*b*) found that CO_2 was the critical volatile factor that stimulates hyphal growth arising from germinating spores of *G. margarita*. The present study did not, however, compare the two root systems with respect to their ability to attract germ tubes by volatile compounds. Using a root organ culture assay, Gemma and Koske (1988) reported chemotropic attraction of *Gigaspora gigantea* germ tubes by host roots but not by nonhost roots. Our results show that sugar beet roots do not produce volatile inhibitor(s), contrary to the hypotheses proposed for certain nonmycorrhizal plants in the genus *Brassica* (Tommerup 1984; El-Atrach *et al.* 1989).

A better explanation for the lack of infection of sugar beet is the inability of their exudates to promote hyphal growth. That these exudates do not inhibit fungal growth indicates that they do not contain fungitoxic compounds. This is reinforced by the fact that the presence of sugar beet roots did not affect fungal development and mycorrhizal infection of carrot roots. In fact, the lack of inhibitory exudates from sugar beet roots is not surprising, since none of the nonmycorhizal plants species in Caryophyllales (including members of Chenopodiaceae, Caryophyllaceae, Polygonaceae, and Amaranthaceae)

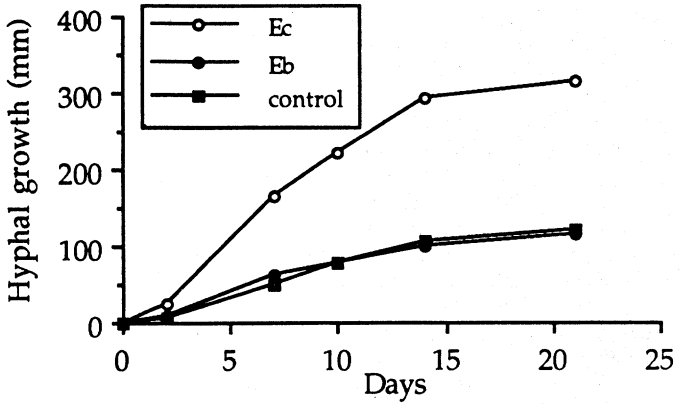

FIG. 4. Average hyphal growth from germinating spores of *G. margarita* in presence of carrot root volatiles without root exudates (control) or with exudates produced by growing roots of carrot (Ec) and sugar beet (Eb).

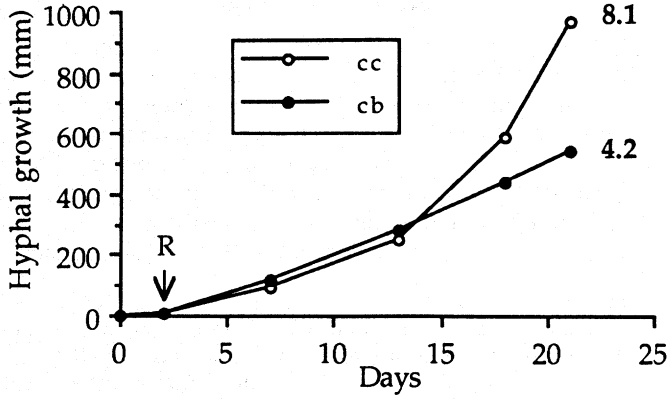

FIG. 5. Average hyphal growth from germinating spores of *G. margarita* in culture with two carrot roots (*cc*) or with one carrot root and one sugar beet root (*cb*). The roots (R) were introduced 2 days after spore germination. The average number of infection units in the carrot root systems on day 21 is indicated beside the respective data points.

have yet been reported to produce inhibitors in relation to VA mycorrhizae. Hypotheses for nonmycorrhizal plants involving inhibitors were always proposed with members of Brassicaceae in the order Capparales (Tester *et al.* 1987). We can assume that since the orders Capparales and Caryophyllales are evolutionarily distinct groups, the mechanisms of "nonmycorrhizalness" are not necessarily identical in both groups (S. M. Schwab, personal communication).

Even if the system of root organ culture involved isolated roots in which the carbon flow may not be similar to that of intact plants, there probably are differences in the quantity and (or) quality of root exudation by the two species (sugar beet and carrot) involved in the differential response of the fungus. Correlations between the degree of mycorrhizal infection and the amount of root exudation were reported (Azcon and Ocampo 1981; Graham *et al.* 1981; Schwab *et al.* 1982; Schwab *et al.* 1984), but they were not always found (Azcon and Ocampo 1984). More recently, studies on different plant systems support the idea that the quality rather than the quantity of exudates is involved in the stimulation of hyphal elongation (Carr *et al.* 1985; Elias and Safir 1987). Glenn *et al.* (1988), after studying the influence of glucosinolate contents of Brassica roots on growth of *Glomus mosseae* and *G. gigantea* in agar, suggested that Brassica roots do not produce a diffusible inhibitor but lack a diffusible growth stimulus normally present near roots of compatible hosts. These latter reports are consistent with our results, suggesting that some nonmycorrhizal plants lack factors that promote mycorrhizal infection rather than producing inhibitory factors.

The factor(s) eliciting hyphal proliferation in the rhizosphere of mycorrhizal plants may not be the decisive factor(s) of infection. Comparing the sequence of stages in the development of infection of *Trifolium subterraneum* L. and *Brassica napus* L., Tommerup (1984) found that all these stages occurred in *B. napus* but at a relatively slow rate. Therefore, root exudates from mycorrhizal plants may act by accelerating infection processes. However, they might also serve as host recognition cues for an endomycorrhizal fungus. Similar active chemical cues in host exudates were reported, and some of them were identified in plant–pathogen associations (Callow *et al.* 1988).

The barriers to the VA mycorrhizal infection of sugar beet roots could be examined further by considering the case where carrot roots are present. The rapid growth of the fungus, initially caused by growth stimulators exudated by carrot and then to biotrophic growth as a result of carrot root infections, was not followed by any form of infection of sugar beet roots. This was surprising, since several papers reported induced infection of roots of nonmycorrhizal plants when they were grown in the presence of a mycorrhizal host (Trinick 1977; Hirrel *et al.* 1978; Ocampo *et al.* 1980; Miller *et al.* 1983; Plenchette and Trouvelot 1986). In the latter study (Plenchette and Trouvelot 1986), 82 cultivars of *B. vulgaris* L. of different origins were tested, and all were infected by *Glomus* spp. in pot culture with leeks as mycorrhizal companion plants. However, although Hirrel *et al.* (1978) found that different species within the Chenopodiaceae could be infected with *Glomus fasciculatus* in the presence of different mycorrhizal plants, they found that *B. vulgaris* and *Atriplex hortensis* L. were exceptions. Despite these contradictions, our results demonstrate that under our conditions, there is at least a second barrier to the process of sugar beet root infection. This barrier may involve cell-to-cell contact between the two organisms at the root surface. Cytochemical studies may confirm the lack of a fibrillar sheath that was found in compatible associations involving ectomycorrhizae and ericoid mycorrhizae (Bonfante-Fasolo and Gianninazzi-Pearson 1982; Piche *et al.* 1988; Lei 1988). Such protein–polysaccharide fibrils are thought to have a role in cell-to-cell recognition or attachment.

The *in vitro* model used in this study, involving host and nonhost transformed root systems and the VA mycorrhizal fungus *G. margarita*, appears to be a promising tool for studies on recognition mechanisms in endomycorrhizal associations. The low virulence of *G. margarita* observed in our conditions and shown by Ocampo *et al.* (1980) indicates that this fungus is particularly sensitive to recognition processes. Our results showed that exudates from the two root systems differed in their ability to stimulate hyphal growth from germinating spores of *G. margarita*. The nonhost root can serve as a negative control for further studies, including the screening for critical growth stimulants in root exudates and the analysis of root–fungal attachment. This latter point is one of our present interests and will be studied using microscopic and cytochemical techniques.

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